Available online at www.sciencedirect.com
ScienceDirect
Science of the Total Environment 371 (2006) 391–401
Science of the Total Environment
An International Journal for Scientific Research into the Environment and its Relationship with Humankind
www.elsevier.com/locate/scitotenv

Correlations of nonylphenol-ethoxylates and nonylphenol with biomarkers of reproductive function in carp (*Cyprinus carpio*) from the Cuyahoga River

Carys L. Mitchelmore [a,*], Clifford P. Rice [b]

[a] *University of Maryland Center for Environmental Science, Chesapeake Biological Laboratory, P.O. Box 38, Solomons, MD 20688, USA*
[b] *U.S. Department of Agriculture, Beltsville Agricultural Research Center, Agricultural Research Service, Environmental Management and Byproducts Utilization Laboratory, Bldg. 001, Rm 219, BARC-West, 10300 Baltimore Avenue, Beltsville MD 20705-2350, USA*

Received 21 July 2006; received in revised form 28 August 2006; accepted 29 August 2006
Available online 19 October 2006

Abstract

Various chemical and biological measures were determined in carp (*Cyprinus carpio*) sampled from seven sites along the Cuyahoga River, Ohio; from the relatively pristine headwaters to the lower portion heavily polluted from various industrial, urban and wastewater treatment plants (WWTP). Levels of nonylphenol (NP), nonylphenol ethoxylates (NPEs; NP1EO, NP2EO) and total NPEs (NP plus the NPEs) in fish increased in a downstream direction, with maximal values observed below the discharge of the Akron WWTP. In female fish there were no significant differences between sites in GSI or levels of vitellogenin (VTG) and 17β-estradiol (E2). However, differences were observed between sites using measures of 11-ketotestosterone (11-KT) and the ratio E2/11-KT. In male fish the highest levels of VTG were observed downstream of the Akron WWTP and a significant correlation (r=85%) between levels of NP and VTG was demonstrated. No site differences were observed in the measures of GSI, E2, 11-KT or the E2/11-KT ratio in male fish. These data suggest that endocrine active chemicals, such as, NP and NPEs are impacting fish downstream of the Akron WWTP; however, further work is warranted to separate linkages to other possible chemical factors in the water.

Keywords: Carp; Fish; Cuyahoga River; Nonylphenol; Biomarkers; Vitellogenin

1. Introduction

Nearly everyone's image of the Cuyahoga River, Ohio, is colored by the infamous fire in 1969. The image is that of an industrial cesspool that starting in the mid-1800s is exactly what it became downstream of Akron and Cleveland. Fires were not unusual on the oil-slicked waters. That one fire became an international symbol of environmental degradation—a rallying cry for the modern environmental movement. Contrary to the lower portion of the Cuyahoga River, the headwaters of the river in Geauga County have remained remarkably pristine (Eco-City, 1998). The middle and lower portions of the river receive industrial and urban wastes

* Corresponding author. Tel.: +1 410 326 7283; fax: +1 410 326 7210.
E-mail address: mitchelmore@cbl.umces.edu (C.L. Mitchelmore).

0048-9697/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.scitotenv.2006.08.049

from the cities of Kent, Cuyahoga Falls, Akron and Cleveland. The Cuyahoga River receives effluent from the Akron Wastewater Treatment Plant (WWTP), composed of both urban and industrial (approximately 10% of the total) inputs. This WWTP during low flow conditions can contribute around 70% of the flow in the river. A further direct WWTP discharge is found at Southerly. For additional information on stream parameters and inputs see Rice et al. (2003) and Ohio EPA (2002). Many chemical contaminants have been reported in the Cuyahoga River (fish, sediment and/or water samples), including heavy metals, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and more recently modest levels of alkylphenol and alkylphenol-ethoxylates (APEs) (Baumann et al., 1991; Rice et al., 2003). Alkylphenol and alkylphenol-ethoxylates are used in industrial processes and in household products due to their surfactant properties and are often found in elevated levels downstream of WWTPs. These surfactants are frequent contaminants in fish, especially those collected from waters near known areas of wastewater and industrial discharges (Ahel et al., 1996; Lye et al., 1999; Rice et al., 2003). Various studies in the Cuyahoga River have shown potentially pollutant-driven disturbances in resident biota, including changes in species diversity and abundance (e.g. Brown and Olive, 1995), enzyme alterations and tumor formation in fish (Henson and Gallagher, 2004). One of the more puzzling recent pollutant-related problems within the Cuyahoga River is the fact that higher-trophic level fish species are not establishing themselves in the middle portion of the river, particularly from the confluence with the Little Cuyahoga to the river mouth. Data collected by the Ohio EPA suggest that a significant source of impairment to fish communities in this region could be coming from the Little Cuyahoga river (Ohio EPA, 2002).

There is mounting evidence that hormone-mimicking pollutants in surface waters are exerting negative effects on fish communities and impacting reproductive processes. One commonly reported impact results from the stimulation of vitellogenin (VTG) production in male fish. Vitellogenin is a yolk precursor protein, which is produced in the liver in response to elevated levels of 17-β-estradiol (E2) and is present at high levels in the blood of female fish. Although the VTG gene is present in male fish it is not expressed so plasma VTG levels in male fish are normally very low or absent. However, various chemical contaminants, including alkylphenols, alkylphenol-ethoxylates, and their metabolites have been reported to mimic estrogen and to stimulate male fish to produce this protein and have, therefore, been classified as endocrine disruptor chemicals (Jobling and Sumpter, 1993; Foran et al., 2000). As male fish are unable to incorporate VTG into eggs a buildup of this protein in the liver and blood stream occurs (Folmar et al., 2001). This may lead to impaired hormone levels, reproductive and/or behavioral impairment (e.g. Cardinali et al., 2004), altered somatic indices (hepatic (HSI) and gonad (GSI) somatic indices) and even organ damage (e.g. to testis, liver and kidney; Simpson et al., 2000). Additionally, the production of this unwanted protein in male fish represents an energetic cost to the organism, which ultimately may impact overall fitness.

The analysis of circulating steroid hormone levels (e.g. 17-β-estradiol; E2 and 11-ketotestosterone; 11-KT) and concentrations of VTG in male fish, have become common biomarkers for monitoring rivers for endocrine-active contaminants. Indeed, elevated levels of VTG and alterations to steroid hormones have been reported in field collected carp (e.g. Lavado et al., 2004), including those collected from sites known to be highly contaminated with alkylphenols (e.g. Garcia-Reyero et al., 2004; Petrovic et al., 2002; Sole et al., 2003). Various laboratories have demonstrated both VTG inductions and alterations in steroid hormones following NPE exposures (e.g. Casini et al., 2002; Rankouhi et al., 2004) although other studies have not (Villeneuve et al., 2002).

This study was conceived and designed by the staff of Ohio EPA and was part of a larger program of sampling and analysis on this river that included organic and inorganic water and sediment pollutant scans. Information about these other data can be obtained from the staff of the Ohio EPA, Groveport, OH 43215 (Ohio EPA, 2002). This study specifically focused on the chemical analyses and biological effects of APEs in river water, sediment and fish tissues. Details on the APE levels in water, sediment and fish (not sex separate) can be found in Rice et al. (2003). These analyses demonstrated that NP and NPEs (NP1 to 3EOs) typically accounted for greater than 90% of the total APEs in each sample, therefore, this subsequent publication presents total (NP and NPE), NP and NPE levels (sex specific) and bioaccumulation factors (BAFs) in the common carp (*C. carpio*). Biological parameters, including levels of VTG and specific hormones (17-β-estradiol, and 11-ketotestosterone) were also assessed in order to determine potential endocrine effects of NP and NPEs in this river.

2. Materials and methods

2.1. Fish collections

Sampling locations consisted of seven sites along a 74-mile length of the Cuyahoga River (see Fig. 1 and

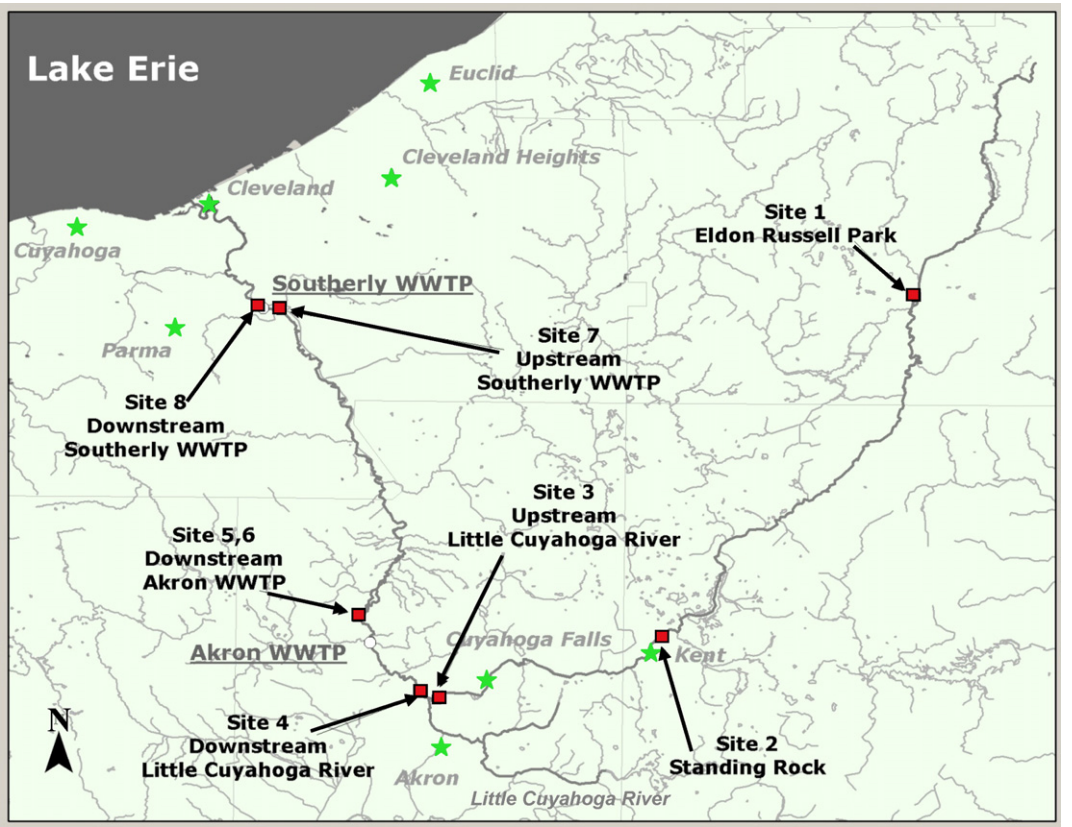

Fig. 1. Location of sampling sites along the Cuyahoga River.

Table 1). All distances are reported as river mile upstream of the point of discharge into Lake Erie. Site 1 was located at the headwaters in Eldon Russell Park and is a relatively pristine site (83.7 river mile). Downstream sites receive anthropogenic inputs from a variety of sources, including discharges from wastewater treatment plants. Site 2 is located at standing rock (55.7 river mile). Site 3 (42.6 river mile) is located upstream of the Little Cuyahoga River which receives a variety of discharges from Akron. To assess the potential impact of these discharges, site 4, is located downstream of the Little Cuyahoga River (38.6 river mile). Sites 5 and 6 were combined and are reported as Site 6 (35.3 river mile), which is located downstream of a major wastewater treatment plant (Akron WWTP). Sites 7 and 8 were chosen to assess the impact of the

Table 1
Biological parameters in male and female fish at each site (mean±S.D.; n=5–6)

Cuyahoga River sites	GSI		CF		% Lipid	
	Male	Female	Male	Female	Male	Female
Site # 1	6.08±1.6	8.86±4.5	1.37±0.1	1.55±0.1	7.18±3.5	9.78±5.7
Site # 2	5.48±0.6	14.14±6.1	1.53±0.1	1.68±0.2	12.40±3.1	12.48±4.6
Site # 3	6.80±2.6	9.53±5.4	1.47±0.1	1.69±0.1	9.63±5.9	7.76±2.9
Site # 4	5.33±1.3	10.27±7.4	1.53±0.2	1.55±0.1	9.93±3.6	9.43±2.9
Site # 5/6	5.39±0.7	15.23±5.1	1.50±0.1	1.55±0.2	13.40±2.3	7.66±2.2
Site # 7	5.47±1.7	12.86±6.6	1.36±0.1	1.49±0.2	8.35±4.3	7.43±4.2
Site # 8	5.79±1.5	14.76±6.1	1.46±0.1	1.44±0.2	9.73±5.8	5.82±2.0

No significant differences in any parameter (GSI = gonadosomatic index, CF = condition factor) were observed in male or female fish (P>0.05).

Southerly WWTP, site 7 being upstream and site 8 being downstream of this WWTP (11.0 and 8.9 river miles, respectively).

Common carp (*C. carpio*) were collected from July 11 to July 13, 2000 using boat mounted pulsed DC electro-fishing gear. Sampling sites consisted of 500 m river segments and live fish were kept in a floating live well until workup. Adult male and female fish ($n=5–6$ of each sex) were sampled at each site. Fish were incapacitated with a blunt instrument or the spinal cord was severed. The length and total weight were recorded. Blood was drawn from the caudal vein though an 18-, 20- or 21-gauge needle using a heparin-treated syringe. Blood was collected into heparinized tubes containing protease inhibitors (aprotinin and phenylmethyl–sulfonyl fluoride, PMSF) and centrifuged to collect the plasma. The plasma was frozen in liquid N_2, stored at −80 °C until analysis. Gonads were dissected and weighed. Following workup each carp was wrapped in aluminum foil, sealed in a plastic bag, placed in dry ice and brought back to the Ohio EPA field facility for placement in a −20 °C freezer. For transport to Beltsville, MD, the fish and sediment samples were shipped in coolers with dry ice under full chain of custody. On arrival they were then placed in a −20 °C freezer until analysis.

2.2. Biological measures and reproductive biomarkers

General biological and health measures were determined for each fish, including, length and weight (whole fish and gonad weight). Gonadal somatic indices were calculated (GSI) as the weight of organ (g)/weight of fish (g)×100. Condition factor was calculated as weight of fish (g)/length^3 (cm) and percentage lipid were also calculated (as detailed in Section 3). Gonads of fish were classified according to four (females) or three (males) stages of sexual maturation, based on analysis of histological slides (carried out by Dr. Paul Stromberg, Ohio State University). Descriptions of the use of this staging method can be found in Goodbred et al. (1997).

Various endocrinological parameters were measured including, circulating steroid levels (17β-estradiol, E2; 11-ketotesterone, 11-KT) and levels of vitellogenin (VTG) in blood plasma. Plasma samples were analyzed for circulating steroids by Dr T.S. Gross (University of Florida, Gainsville, FL) using radioimmunoassay (RIA) procedures as detailed in (Goodbred et al., 1997). Vitellogenin concentrations in plasma were assayed and quantified by capture enzyme-linked immunosorbant assay (ELISA) in the laboratory of Dr. N. Denslow (University of Florida, Gainsville, FL) as previously described (Folmar et al., 2001).

2.3. Carp chemical analyses and bioaccumulation factors

Details of the sample homogenization, extraction and clean-up of carp tissues together with details of the instrumental methods used to determine NP and NPE's in fish tissues are described in a previous publication (Rice et al., 2003). Briefly the method involved accelerated solvent extraction, aminopropyl solid phase clean-up, and separation and analyses of individual homologue mixtures of nonylphenol (NP), nonylphenol ethoxylate (NP1EO), and nonylphenol-2-ethoxylate (NP2EO) utilizing HPLC, normal phase separation, and fluorescence detection. Selected sample extracts were also analyzed using HPLC reverse-phase column separation and triple quadrupole mass spectrometry which was used to confirm both qualitatively and quantitatively the fluorescence detection findings.

Using the data available from this study and the NPE data for sediment and water contained in Rice et al. (2003), it was possible to estimate bioaccumulation factors for fish via water (BAFs) and via sediment (BSAF). These bioaccumulation factors were determined for the NP group, and by degree of ethoxymer substitution from the phenol form to the 2-ethoxymer. To calculate the BAFs the following equation was used, C_B/C_W, where C_B is the concentration of the analyte in the fish on a wet weight basis and C_W is the concentration of the analyte in the water (since these compounds are predominantly in the dissolved state, e.g., $K_{ow}<5$ (Gobas and Morrison, 2000). The sediment accumulation factors were calculated using the following equation, C_{Bd}/C_S, (Hellou et al., 1995) where C_{Bd} is the fish concentration expressed on a dry weight basis (assumed fish had 76.3% water (U.S. FDA, 1989)) and C_S is the sediment concentration expressed on a dry weight basis.

2.4. Statistical analyses

Data were analyzed using Minitab statistical software. Data were assessed for normality (Shapiro-Wilk) and assumptions of equal variance and where necessary the data was transformed. An alpha value for 0.05 was used in all analyses. One-way analyses of variance (ANOVAs) were performed and when significant differences were found multiple comparison tests (Tukey's studentized range test, HSD) were used to assess differences between sites among the parameters tested. To

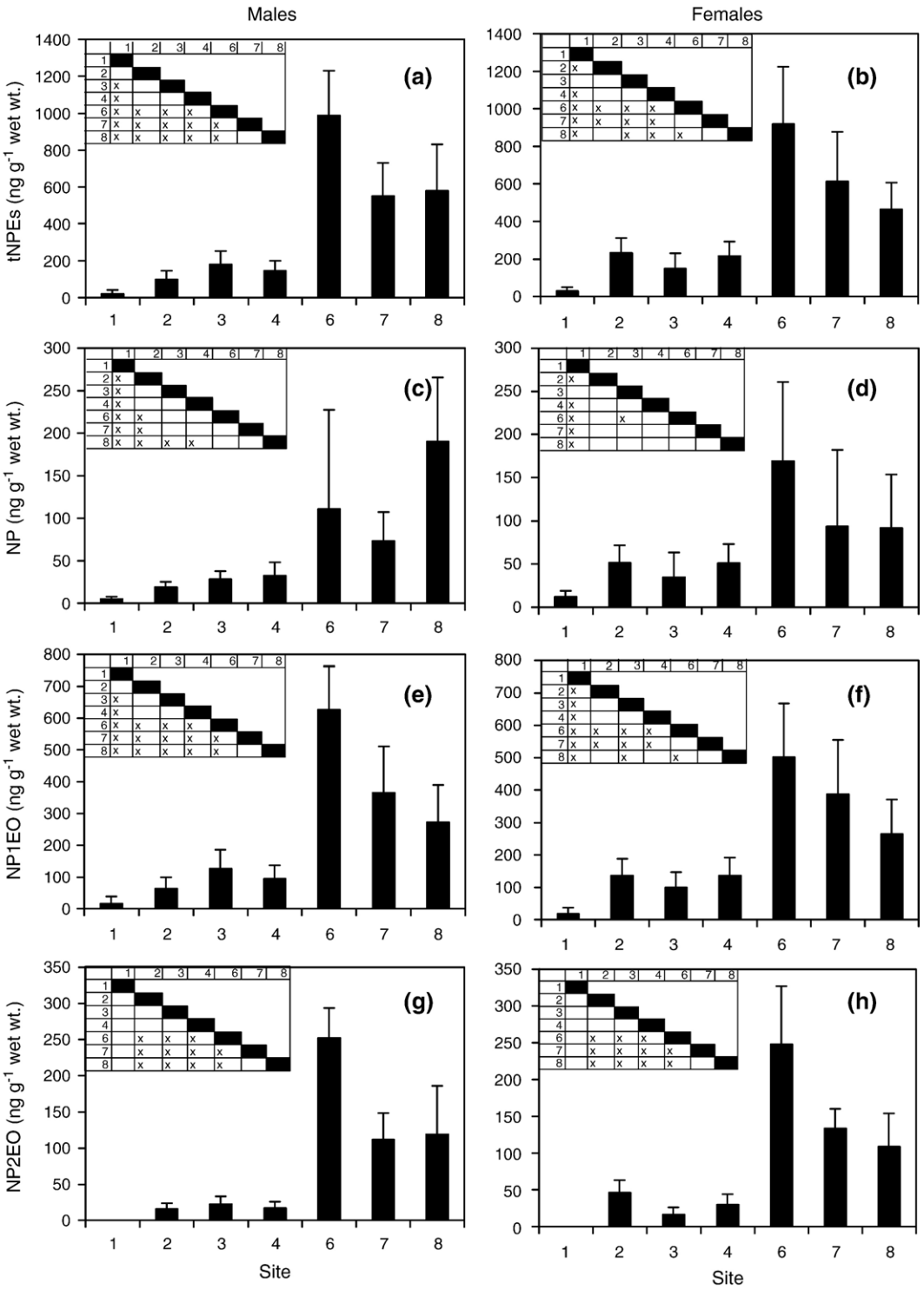

Fig. 2. Concentrations of total NPEs, NP, NP1EO and NP2EO in male and female carp at each sampling site (averages ± stdev, $n = 5–6$). Graphs for males (a, c, e, g) and females (b, d, f, h) are given for each chemical (tNPEs, NP, NP1EO and NP2EO respectively). Statistical differences reported in table; x signifies site means that are different at the 0.05 significance level.

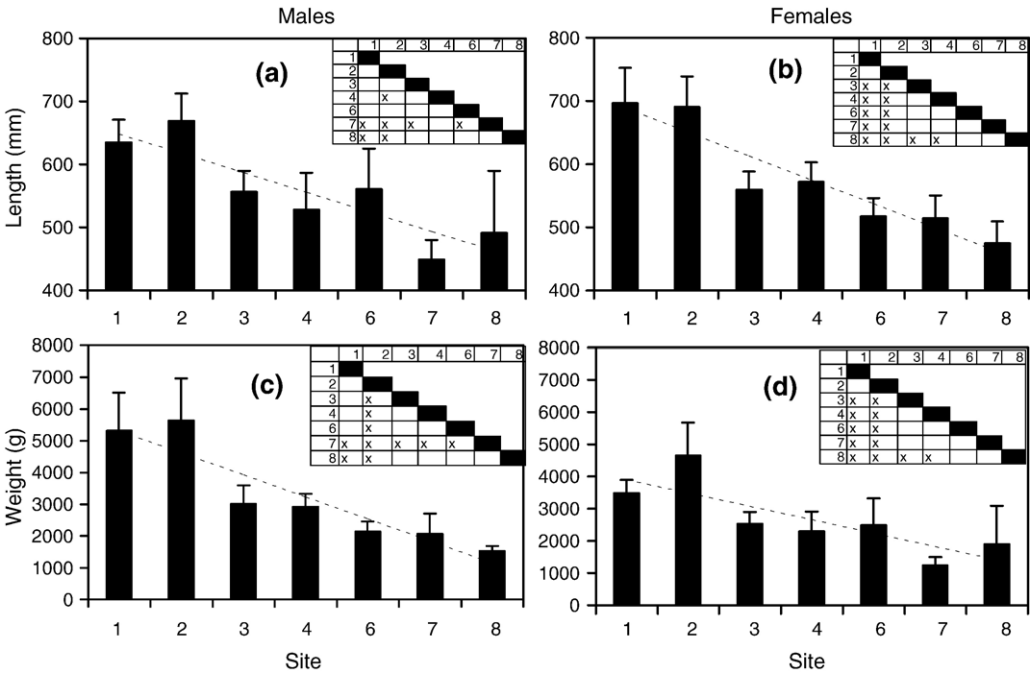

Fig. 3. Length and weight of fish at each sampling site (means±S.D., $n=5–6$). Statistical differences reported in table; x signifies site means that are different at the 0.05 significance level.

determine relationships between contaminant and biological parameters Pearson's product moment correlation analyses were performed and data reported as *r*-values.

3. Results

3.1. Chemical analyses and bioaccumulation factors

Mean levels of total APEs (both NPE and OPEs), nonylphenol (NP), nonylphenol monoethoxylate (NP1EO) and nonylphenol diethoxylate (NP2EO) in carp tissues have previously been reported, although in these analyses fish were not separated by sex (Rice et al., 2003). Fig. 2 details the sex-specific concentrations of total NPEs (tNPEs), and individual homologues, in female and male carp tissues. Similar levels of total NPEs were observed in both male and female fish (Fig. 2a, b) with the lowest levels in both observed at the uppermost site (site 1). A peak in levels was observed at site 6, downstream of the Akron WWTP and elevated levels were also observed at sites 7 and 8. Fig. 2c, d demonstrate the similar levels of NP between the sexes. Again the lowest levels were observed at site 1 and elevated levels were observed at sites 6–8. Similar profiles (low levels at sites 1 and 2 and elevated levels at sites 6–8) were also observed with NP1EO and NP2EO levels in both male and female fish (see Fig. 2e, f and g, h respectively).

The average values for the BAF for the NPE groups were as follows: NP — 280, NP1EO — 1713, NP2EO —

Table 2
Percentage of fish at the different sexual maturation stages at each sampling site (mean±S.D.; $n=5–6$)

CuyahogaRiver sites	Stage I		Stage II		Stage III		Unclassified	
	Male	Female	Male	Female	Male	Female	Male	Female
Site # 1	0	0	83	60	17	40	0	0
Site # 2	0	0	100	80	0	20	0	0
Site # 3	0	17	100	33	0	50	0	0
Site # 4	0	33	100	17	0	50	0	0
Site # 5/6	0	0	0	20	83	80	17	0
Site # 7	0	0	33	17	66	50	0	33
Site # 8	17	0	0	17	83	67	0	17

Table 3
Steroid hormone levels in male and female fish at each site (mean±S.D.; n=5–6)

River sites	E2 (pg ml^{-1})		11-KT (pg ml^{-1})		E2/11-KT		VTG (mg ml^{-1})	
	Male	Female	Male	Female	Male	Female	Male	Female
Site # 1	240.5±95	597.6±261	566.8±225	474.0±193	0.46±0.2	1.55±1.1	0.0485±0.028	2.50±0.9
Site # 2	460.2±231	904.6±507	794.3±297	263.0±118	0.64±0.3	3.68±2.1	0.0455±0.029	1.84±0.5
Site # 3	665.5±314	443.3±333	971.0±474	891.2±518	1.04±1.0	0.79±1.1	0.0267±0.013	2.38±1.1
Site # 4	729.7±422	781.8±552	995.3±584	674.3±369	1.13±1.1	1.62±1.3	0.0373±0.023	2.97±1.3
Site # 5/6	351.2±169	849.8±462	976.5±256	358.8±53	0.40±0.3	2.37±1.3	0.0885±0.075	2.44±1.1
Site # 7	639.3±713	977.0±614	913.5±1021	1558.5±1107*	0.77±0.7	2.97±4.6	0.0372±0.032	1.94±0.9
Site # 8	285.5±151	667.0±352	953.8±514	251.2±84	0.31±0.1	2.86±1.6#	0.0554±0.042	1.38±0.6

No significant differences between sites were observed in levels of E2 or VTG, or levels of 11-KT and E2/11-KT ratios in male fish. In female fish differences between sites were observed in levels of 11-KT and E2/KT (P=0.006 and 0.045 respectively). *; site 7 is significantly different (P<0.05) to sites 2 and 8. #; site 3 is significantly different (P<0.05) than site 8.

693. The sediment BSAFs were as follows: NP — 1.5, NP1EO — 4.4 (Site 6 was excluded as an outlier) and NP2EO — 2.4. The variation between averaged BSAF values for NP and NP2EO did not vary much from site to site, e.g., relative standard deviations (rsd) of less than 0.5. However the rsd values for the BSAF for NP1EO was 1.9. Using a statistical outlier test it was revealed that the BSAF value at site 6 was an outlier and could be excluded from the mean BSAF calculation. Site #6 is located near the outfall for the Akron WWTP, which is the major APEO discharger to this river, and fish accumulations probably are more influenced by aqueous phase loading here than from exposures from the sediment, thus it is logical that BSAF values might be less reliable here.

3.2. Biological parameters

Despite the attempt to obtain fish of similar sizes we found significant differences in length and weight of fish were observed at each sampling location (see Fig. 3, ANOVA P<0.05). Higher values in both parameters were observed at the uppermost sites and values decreased in a downstream direction in both female and male fish (r=65–88%). We found no significant differences between sites in measures of GSI, condition factor or percentage lipid (ANOVA P>0.05; see Table 1) and no significant correlation in these parameters with NPEs were observed (r<10%). Comparisons were made between lipid content of each fish and total NPE concentrations since alkylphenols are considered to be moderately lipophilic. The average lipid content for the carp (106 fish) was 9.3% with a standard deviation of 4.2%. Neither the concentration of any of the analytes (e.g. NPEs r=2.9%) nor site location consistently correlated with the lipid levels in the fish, demonstrating that the concentration of tNPEs, NP, NPEs does not seem to be lipid level dependant.

3.3. Histopathology and reproductive biomarkers

Histopathological evaluations were carried out to determine the stage of reproductive development for each fish and are detailed in Table 2. A significant difference in steroid hormone profiles and VTG levels occur in fish based on stage of development (see Goodbred et al., 1997), therefore, female and male fish at stage 1 in development were excluded from our statistical analyses. There was a significant shift to higher stages of sexual maturity when fish upstream of site 6 are compared with those downstream. For the male fish, the majority (95.8%) are in stage II in the upstream group although in the downstream group this shifted to 77% of the fish being in stage III. For the female fish the majority in the upstream group were equally split between stage II and III (48 and 40% respectively), whereas in the downstream groups the majority were in stage III (66%).

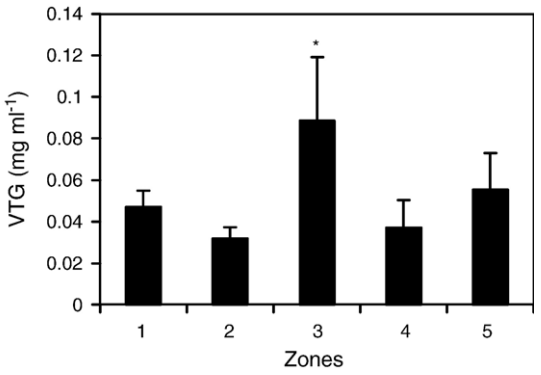

Fig. 4. Levels of vitellogenin in male fish from each sampling zone (means±S.D., n=5–12). Zone 1 (sites 1 and 2), zone 2 (sites 3 and 4), zones 3 (site 5/6), zone 4 (site 7) and zone 5 (site 8). *; signifies a significant difference between zones 1, 2, 4 and 3 at P<0.05.

In the present study with male fish no significant differences (ANOVA $P>0.05$) were observed between sites in the concentrations of E2, 11-KT or the ratio of E2/11-KT (see Table 3) and no significant correlations with levels of NPEs were observed (r values all<10%). Levels of VTG in male fish in this study were highest at site 6, although this was not significantly different compared with the other sites (ANOVA, $P>0.05$; Table 1). This result may be in part due to the low sample size ($n=5$–6) of male fish analyzed at each site as variability in the VTG levels between individual fish was high (particularly site 6). Therefore, to increase sample size, fish were placed into zones based on location of pollutant inputs (groups of sites as follows; sites 1 and 2 = zone 1, sites 3 and 4 = zone 2, site 6 = zone 3, site 7 = zone 4 and site 8 = zone 5). Higher and statistically significant (compared with zone 2, $P>0.02$) levels of VTG were observed at zone 3 downstream of the Akron WWTP (Fig. 4). Correlations between levels of NP and NPEs and male VTG levels in our study were low (i.e. total NPEs (tNPEs) $r=17\%$, NP1EO $r=12\%$ and NP2EO $r=11\%$), the highest correlation being with NP levels (see Fig. 5a; $r=30\%$). However, when levels of NP were placed into 5 rank orders (see Fig. 5b) and the average values for VTG and mean NP levels in each rank are compared then there is a highly significant correlation ($r=97\%$) between NP and VTG levels.

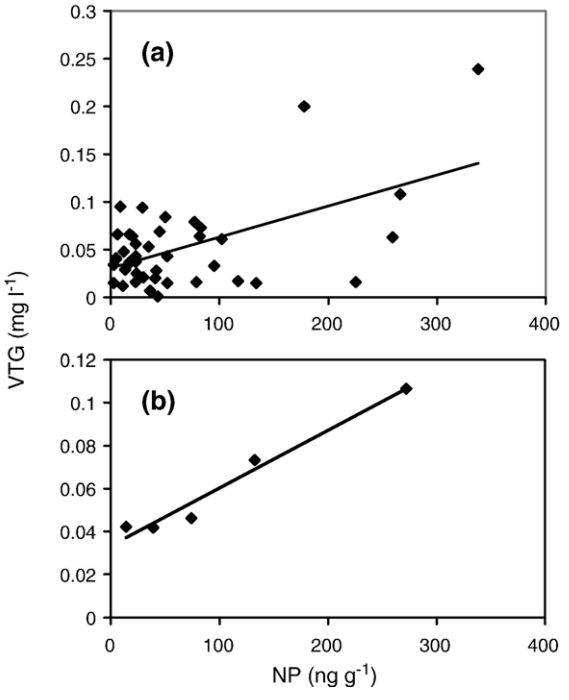

Fig. 5. Correlation between levels of VTG and NP in male fish for (a) individual fish at all sites ($r=31\%$) and (b) using ranked scores of NP levels; 0–25, 25–50, 50–100, 100–200 and >200 ng g^{-1} NP assigned to ranks 1–5 respectively, data reported for each rank as means±S.D., $n=4$–18 ($r=98\%$).

Females showed no differences between sites in levels of VTG or E2 (Table 3). However, there were significant differences in levels of 11-KT and the ratio of E2/11-KT between sites, although there was no correlation or patterns observed with levels of NPEs (r all<10%).

4. Discussion

A major advantage offered by this study is the fact that biological responses in individual fish are compared to accumulated levels of the NPEs in each fish and not external concentrations which is typical of the majority of research published in this area. Exposure levels, especially aqueous concentrations are prone to wide fluctuations depending upon when sampling is done and existing flow. Although the measured levels of total NPEs (tNPEs) in water samples collected from sites 1–6 correlated well ($r=39\%$) with total levels of tNPEs in carp (pooling male and female data; see Rice et al. (2003)). No correlation was observed between fish tNPEs and sediment concentrations ($r=2\%$). The concentrations in the fish will integrate exposure over time, the different potential routes of exposure and more accurately reflect the concentrations available to act at the biological target sites. As discussed in the previous publication (Rice et al., 2003) these NP levels are higher compared with some carp studies (e.g. Bennie et al., 1998; Keith et al., 2001) but moderate compared to carp data reported by others (Datta et al., 2002). In addition, the levels of the NP ethoxylates have been described as some of the highest reported for fish in United States waters (see Rice et al., 2003).

Comparing the BAF values to published numbers; it appears that our values for NP are within expected ranges. The recent reviews by Staples et al. (2004) and Servos (1999) indicate that most accumulation data are available for NP, where laboratory-controlled uptake studies yielded BCFs in the 200 to 300 range and nearly all their field-derived accumulation factors (BAFs) (NP and NP-ethoxymers) were lower than their BCFs. We calculated some additional BAFs from more recent publications and came up with the following values: Snyder et al. (2001) data for carp from Lake Mead produced an average BAF for nonylphenol of 344 and for NP1EO the value was 60; using data from Lye et al. (1999) for flounder from Tyne estuary the calculated BAFs were 230 for NP; and taking Blackburn et al.

(1999) data for several fish species a range in BAFs of about 60 for NP to 53–930 for the sum of the 1 and 2 NP-ethoxymers were calculated. When comparing these data to our values only the NP results were similar, and most published ethoxylate data were low relative to the BAFs calculated here. The calculated accumulation factors for the APEs from the sediment were less than for the water; however, they were all greater than one, indicating sediment may likely be a source. These BSAF values were highest for the 1 ethoxymer, 7.2 NP1EO. There are no data on BSAFs for APEs in fish to check these against. Hellou et al. (1995) determined BSAF values for a range of PAHs and some of these compounds had similar BSAF to those observed here for the APEs.

In some studies levels of NPEs have been negatively correlated with growth in fish species (see Staples et al., 2004 for a review). We did not observe any significant correlations of length or weight with NP or NPEs in this study ($r<10\%$). The water concentrations of NP and NPEs reported by Rice et al. (2003) at our most contaminated study sites are at least an order of magnitude lower than those that were used where growth inhibitions (Staples et al., 2004) were observed. However, potential routes of exposure of our fish to NP and NPEs also may have arisen via sediment and food sources. Decreased GSI ratios have often been observed in male carp following exposure to WWTP effluent (Diniz et al., 2005; Lavado et al., 2004), although not always (Carballo et al., 2005). Laboratory exposures of fish to NP or NPEs have also shown similar results (e.g. Le Gac et al., 2001; Li and Wang, 2004). In a study by Villeneuve et al. (2002) no alterations in GSI ratio were observed when carp were exposed to similar levels of NP. In our study it appears that despite larger (weight and length) fish being observed in the upstream group, sexual maturity is greater in the downstream sites. Therefore, there may be impacts to sexual maturity at these downstream sites.

Circulating levels of male hormones have routinely been used as biomarkers (i.e. testosterone (T) and 11-KT). 11-ketotestosterone (11-KT) is generally regarded as the most important form of androgen (and is closely correlated with T levels; see Goodbred et al., 1997) and was, therefore, analyzed in this study. Studies have shown depressed levels of T (or 11-KT) downstream of WWTP (e.g. Folmar et al., 1996; Leatherland, 1992), including in carp (Lavado et al., 2004). However, in carp exposed to NP no alterations in levels of 11-KT were observed (Villeneuve et al., 2002). Exposure to NP has been shown to elevate levels of E2 in some fish species (i.e. Giesy et al., 2000), although not in carp (Villeneuve et al., 2002) and in Atlantic Salmon decreased levels of E2 were observed (Arukwe et al., 1997). The ratio of E2/11-KT has also been described as a very sensitive biomarker as the balance of these two hormones is critical for a number of developmental processes (see Folmar et al., 1996). Considerable variations in plasma sex steroid concentrations between individuals were observed at each site, which is similar to an extensive survey of feral carp from a variety of U.S. rivers (Goodbred et al., 1997). E2 levels range from 241 to 730, which is in the range reported in feral carp in other studies (Goodbred et al., 1997; Sole et al., 2003). Similarly our levels of 11-KT (567–995) are also within ranges reported in other studies (Goodbred et al., 1997). Goodbred et al. (1997) reported significant correlations between levels of E2 and 11-KT, although we observed a low correlation with E2 ($r=11\%$) although this was higher ($r=40\%$) with the E2/11-KT ratio. This ratio is usually <1.0 in male fish (see Goodbred et al., 1997) and as shown in Table 3 our data follows this pattern. An increase in the E2/11-KT ratio in male carp from polluted sites has been demonstrated (Folmar et al., 1996), although we did not observe this.

The most common endocrine biomarker used in male fish is that of circulating levels of VTG. Many studies have shown elevated levels of VTG in carp downstream of WWTPs (Lavado et al., 2004; Li and Wang, 2004) although some have not (Villeneuve et al., 2002). Carp exposed to NPs in the laboratory have also been shown to upregulate VTG levels (Casini et al., 2002). In a report by Petrovic et al. (2002) significant correlations between APEs (in water, however, they were not analyzed in fish tissues) and VTG levels in carp downstream of a WWTP were demonstrated ($r=84\%$). Levels of VTG in male fish in our study were comparable to those documented in a similar study (e.g. Goodbred et al., 1997). Correlations between levels of NP and NPEs and male VTG levels in our study were low with the highest correlation being with NP levels. However, when levels of NP were placed into 5 rank orders and the average values for VTG and mean NP levels in each rank are compared then there is a highly significant correlation between NP and VTG levels comparable to the Petrovic et al. (2002) study. Comparing the correlation between levels of NPEs and VTG in male fish at site 6 only (highest VTG site) resulted in r values (NP2EO 8%, NP1EO 50% and tNPEs 70%), with a highly significant correlation (85%) observed between levels of NP and VTG (compared with only a 9% correlation of NP levels and VTG at site 8). Correlations of VTG levels and GSI have been observed (e.g. Cardinali et al., 2004), however we did not observe any significant correlation in this study ($r=<10\%$).

Although not as well documented as for male responses, alterations in normal VTG levels in females have also been proposed as biomarkers of estrogenic exposure (Kime et al., 1999). The steroid hormone and VTG levels in female fish, like in the males, were highly variable between fish. Levels of E2, 11-KT and VTG were within the ranges reported in other studies of field collected female carp (e.g. Goodbred et al., 1997; Sole et al., 2000). E2/11-KT ratios at most sites were above 1.0, which is similar to other studies (e.g. Goodbred et al., 1997).

5. Conclusions

Accumulation of NP and NPEs are evident in both male and female fish collected from downstream sites of the Cuyahoga River, and appear to correlate well with location of WWTPs. However, limited sampling sizes and variations between individual fish made definitive observations of endocrine impact difficult. These endocrine biomarkers are often complicated by natural variables, including water temperature (Sole et al., 2003) and seasonality issues (Sole et al., 2002), although water temperatures at each site in the current study did not vary, the one-time sampling effort (late spawning period) may have attributed to the lack of differences observed between sites. Indeed in a study by Higashitani et al. (2003) in which carp were sampled at varying times throughout the year significant elevations in VTG in male carp were only observed in the Spring. In the present study evidence of endocrine disruption using steroid hormone and VTG levels was limited to elevations in VTG production in male fish downstream of the WWTP at site 6, which correlated well with NP levels at that site. However, many other organic chemical contaminants have also been detected downstream of WWTPs and may be impacting the carp. Further studies using increased sample sizes, repeated sampling throughout the year, and a better accounting of other VTG inducing chemicals in the water (i.e. estradiol and ethinylestradiol) will aid future work in determining estrogenic effects in Carp from the Cuyahoga River.

Acknowledgements

This work was completed with partial funding from the State of Ohio, Ohio Environmental Protection Agency. Trust Fund Cooperative Agreement No. 58-1270-0-051 with Ohio Environmental Protection Agency, entitled: Determination of Alkylphenols and Alkylphenol Ethoxylates in Fish, Sediment, and Water from the Cuyahoga River. Termination 10/31/2001. The steroid hormone assays were carried out by Dr T. Gross and the VTG analyses in the laboratory of Dr Nancy Denslow. Special thanks to Kerry Sefton for preparing the Cuyahoga map and to Dr Chris Rowe for statistical advice.

References

Ahel M, Schaffner C, Giger W. Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment.3. Occurrence and elimination of their persistent metabolites during infiltration of river water to groundwater. Water Res 1996;30:37–46.

Arukwe A, Forlin L, Goksoyr A. Xenobiotic and steroid biotransformation enzymes in Atlantic salmon (*Salmo salar*) liver treated with an estrogenic compound, 4-nonylphenol. Environ Toxicol Chem 1997;16:2576–83.

Baumann PC, Mac MJ, Smith SB, Harshberger JC. Tumor frequencies in walleye (*Stizostedion-vitreum*) and brown bullhead (*Ictalurus-nebulosus*) and sediment contaminants in tributaries of the Laurentian Great-lakes. Can J Fish Aquat Sci 1991;48:1804–10.

Bennie DT, Sullivan CA, Lee HBMRJ. Water Qual Res J Can 1998;33:231–52.

Blackburn MA, Kirby LJ, Waldock MJ. Concentrations of alkyphenol poly-ethoxylates entering UK estuaries. Mar Pollut J 1999;38:109–18.

Brown BJ, Olive JH. Diatom communities in the Cuyahoga River (USA) — changes in species composition between 1974 and 1992 following renovations in waste-water management. Ohio J Sci 1995;95:254–60.

Carballo M, Aguayo S, de la Torre A, Munoz MJ. Plasma vitellogenin levels and gonadal morphology of wild carp (*Cyprinus carpio* L.) in a receiving rivers downstream of sewage treatment plants. Sci Total Environ 2005;341:71–9.

Cardinali M, Maradonna F, Olivotto I, Bortoluzzi G, Mosconi G, Polzonetti-Magni AM, et al. Temporary impairment of reproduction in freshwater teleost exposed to nonylphenol. Reprod Toxicol 2004;18:597–604.

Casini S, Fossi MC, Mori G, Bjornstad A. Vitellogenin induction in *Cyprinus carpio* treated with 17 β-estradiol and 4-nonylphenol. Environ Monit Assess 2002;75:235–9.

Datta S, Loyo-Rosales JE, Rice CP. A simple method for the determination of trace levels of alkylphenolic compounds in fish tissue using pressurized fluid extraction, solid phase cleanup, and high-performance liquid chromatography fluorescence detection. J Agric Food Chem 2002;50:1350–4.

Diniz MS, Peres I, Pihan JC. Comparative study of the estrogenic responses of mirror carp (*Cyprinus carpio*) exposed to treated municipal sewage effluent (Lisbon) during two periods in different seasons. Sci Total Environ 2005;349:129–39.

Eco-City. The greater Cleveland environmental book. Cleveland: Eco-City; 1998.

Folmar LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, et al. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environ Health Perspect 1996;104:1096–101.

Folmar LC, Gardner GR, Schreibman MP, Magliulo-Cepriano L, Mills LJ, Zaroogian G, et al. Vitellogenin-induced pathology in male summer flounder (*Paralichthys dentatus*). Aquat Toxicol 2001;51:431–41.

Foran CM, Bennett ER, Benson WH. Exposure to environmentally relevant concentrations of different nonylphenol formulations in Japanese medaka. Mar Environ Res 2000;50:135–9.

Garcia-Reyero N, Raldua D, Quiros L, Llaveria G, Cerda J, Barcelo D, et al. Use of vitellogenin mRNA as a biomarker for endocrine

disruption in feral and cultured fish. Anal Bioanal Chem 2004;378:670–5.

Giesy JP, Pierens SL, Snyder EM, Miles-Richardson S, Kramer VJ, Snyder SA, et al. Effects of 4-nonylphenol on fecundity and biomarkers of estrogenicity in fathead minnows (*Pimephales promelas*). Environ Toxicol Chem 2000;19:1368–77.

Gobas FAC, Morrison HA. Bioconcentration and biomagnification in the aquatic environment. In: Boethling RS, Mackay D, editors. Handbook of property estimation methods for chemicals, environmental and health sciences. Boca Raton, London: Lewis Publishers; 2000. p. 189–231.

Goodbred SL, Gilliom RJ, Gross TS, Denslow NP, Bryant WB, Schoeb TR. Reconnaissance of 17β-estradiol, 11-ketotestosterone. Vitellogenin and gonad histopathology in common carp of United States streams: potential for contaminant-induced endocrine disruption. OF 96-0627 U.S. Sacramento, CA, USA: Department of the Interior—U.S. Geological Survey; 1997. p. 48.

Hellou J, Mackay D, Fowler B. Bioconcentration of polycyclic aromatic-compounds from sediments to muscle of finfish. Environ Sci Technol 1995;29:2555–60.

Henson KL, Gallagher EP. Glutathione *s*-transferase expression in pollution-associated hepatic lesions of brown bullheads (*Ameiurus nebulosus*) from the Cuyahoga River, Cleveland, Ohio. Toxicol Sci 2004;80:26–33.

Higashitani T, Tamamoto H, Takahashi A, Tanaka H. Study of estrogenic effects on carp (*Cyprinus carpio*) exposed to sewage treatment plant effluents. Water Sci Technol 2003;47:93–100.

Jobling S, Sumpter JP. Detergent components in sewage effluent are weakly estrogenic to fish — an in-vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquat Toxicol 1993;27:361–72.

Keith TL, Shyder SA, Naylor CG, Staples CA, Summer C, Kannan K, et al. Identification and quantitation of nonylphenol ethoxylates and nonylphenol in fish tissues from Michigan. Environ Sci Technol 2001;35:10–3.

Kime DE, Nash JP, Scott AP. Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. Aquaculture 1999;177:345–52.

Lavado R, Thibaut R, Raldua D, Martin R, Porte C. First evidence of endocrine disruption in feral carp from the Ebro River. Toxicol Appl Pharmacol 2004;196:247–57.

Leatherland JF. Endocrine and reproductive function in Great Lakes salmon. In: Colborn T, Clement C, editors. Chemically induced alterations in sexual and functional development: the wildlife/human connection. Princeton, NJ: Princeton Scientific Publishing; 1992. p. 129–46.

Le Gac F, Thomas JL, Mourot B, Loir M. *In vivo* and *in vitro* effects of prochloraz and nonylphenol ethoxylates on trout spermatogenesis. Aquat Toxicol 2001;53:187–200.

Li M-H, Wang Z-R. Effect of nonylphenol on plasma vitellogenin of male adult guppies (*Poecilia reticulata*). Environ Toxicol 2004;20:53–9.

Lye CM, Frid CLJ, Gill ME, Cooper DW, Jones DM. Estrogenic alkylphenols in fish tissues, sediments, and waters from the U.K. Tyne and Tees Estuaries. Environ Sci Technol 1999;33:1009–14.

Ohio EPA. Preliminary assessment/site inspection (PA/IS) sampling investigation final report for Cuyahoga River, a study of biomarkers, fish, sediment and surface water of the Cuyahoga River. USEPA ID: OHS FND 508 019; 2002. 20 pp.

Petrovic M, Sole M, de Alda MJL, Barcelo D. Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: integration of chemical analysis and biological effects on feral carp. Environ Toxicol Chem 2002;21:2146–56.

Rankouhi TR, Sanderson JT, van Holsteijn I, van Leeuwen C, Vethaak AD, van den Berg M. Effects of natural and synthetic estrogens and various environmental contaminants on vitellogenesis in fish primary hepatocytes: comparison of bream (*Abramis brama*) and carp (*Cyprinus carpio*). Toxicol Sci 2004;81:90–102.

Rice CP, Schmitz-Afonso I, Loyo-Rosales JE, Link E, Thoma R, Fay L, et al. Alkylphenol and alkylphenol-ethoxylates in carp, water, and sediment from the Cuyahoga River, Ohio. Environ Sci Technol 2003;37:3747–54.

Servos MR. Review of the aquatic toxicity, estrogenic responses and bioaccumulation of alkylphenols and alkylphenol polyethoxylates. Water Qual Res J Can 1999;34:123–77.

Simpson MG, Parry M, Kleinkauf A, Swarbreck D, Walker P, Leah RT. Pathology of the liver, kidney and gonad of flounder (*Platichthys flesus*) from a UK estuary impacted by endocrine disrupting chemicals. Mar Environ Res 2000;50:283–7.

Snyder SA, Keith TL, Naylor CG, Staples CA, Giesy JP. Identification and quantitation method for nonylphenol and lower oligomer nonylphenol ethoxylates in fish tissues. Environ Toxicol Chem 2001;20:1870–3.

Sole M, Castillo M, de Alda MJL, Porte C, Barcelo D. Estrogenicity determination in carp, *Cyprinus carpio*: a laboratory and field approach. Analusis 2000;28:783–8.

Sole M, Barcelo D, Porte C. Seasonal variation of plasmatic and hepatic vitellogenin and EROD activity in carp, *Cyprinus carpio*, in relation to sewage treatment plants. Aquat Toxicol 2002;60:233–48.

Sole M, Raldua D, Piferrer F, Barcelo D, Porte C. Feminization of wild carp, *Cyprinus carpio*, in a polluted environment: plasma steroid hormones, gonadal morphology and xenobiotic metabolizing system. Comp Biochem Physiol C Comp Pharmacol Toxicol 2003;136:145–56.

Staples C, Mihaich E, Carbone J, Woodburn K, Klecka G. Hum Ecol Risk Assess 2004;10:999–1017.

U.S. Food and Drug Administration Pesticide Analytical Manual. Washington D.C.: U.S. GPO; 1989.

Villeneuve DL, Villalobos SA, Keith TL, Snyder EM, Fitzgerald SD, Giesy JP. Effects of waterborne exposure to 4-nonylphenol on plasma sex steroid and vitellogenin concentrations in sexually mature male carp (*Cyprinus carpio*). Chemosphere 2002;47:15–28.